Faculty of Resource Science and Technology

PRODUCTION OF PROTEASE FROM *Aspergillus niger* UNDER SOLID STATE FERMENTATION USING DIFFERENT AGRO WASTE AS SUBSTRATE

Edelisa Jenny Radius

(51650)

Bachelor of Science with Honours
(Resource Biotechnology)
2018

UNIVERSITI MALAYSIA SARAWAK

Grade: _____

Please tick (√)
Final Year Project Report ☑
Masters ☐
PhD ☐

DECLARATION OF ORIGINAL WORK

This declaration is made on the 30 day of MAY year 2018

Student's Declaration:

I EDELISA JENNY RADIUS, 51650, FACULTY OF RESOURCE SCIENCE AND TECHNOLOGY.
(PLEASE INDICATE NAME, MATRIC NO. AND FACULTY) hereby declare that the work entitled, PRODUCTION OF PROTEASE FROM Aspergillus niger UNDER SOLID STATE FERMENTATION USING DIFFERENT AGRO WASTE AS SUBSTRATE is my original work. I have not copied from any other students' work or from any other sources with the exception where due reference or acknowledgement is made explicitly in the text, nor has any part of the work been written for me by another person.

8.06.2018
Date submitted

EDELISA JENNY RADIUS (51650)
Name of the student (Matric No.)

Supervisor's Declaration:

I, ROSMAWATI SAAT (SUPERVISOR'S NAME), hereby certify that the work entitled, PRODUCTION OF PROTEASE FROM Aspergillus niger UNDER SOLID STATE FERMENTATION USING DIFFERENT AGRO WASTE AS SUBSTRATE (TITLE) was prepared by the aforementioned or above mentioned student, and was submitted to the "FACULTY" as a * partial/full fulfillment for the conferment of BACHELOR OF SCIENCE WITH HONORS (RESOURCE BIOTECHNOLOGY) (PLEASE INDICATE THE DEGREE TITLE), and the aforementioned work, to the best of my knowledge, is the said student's work

Received for examination by: ROSMAWATI SAAT (Name of the supervisor) Date: 30/5/2018

I declare this Project/Thesis is classified as (Please tick (√)):

☐ **CONFIDENTIAL** (Contains confidential information under the Official Secret Act 1972)*
☐ **RESTRICTED** (Contains restricted information as specified by the organisation where research was done)*
☑ **OPEN ACCESS**

I declare this Project/Thesis is to be submitted to the Centre for Academic Information Services and uploaded into UNIMAS Institutional Repository (UNIMAS IR) (Please tick (√)):

☑ **YES**
☐ **NO**

Validation of Project/Thesis

I hereby duly affirmed with free consent and willingness declared that this said Project/Thesis shall be placed officially in the Centre for Academic Information Services with the abide interest and rights as follows:

- This Project/Thesis is the sole legal property of Universiti Malaysia Sarawak (UNIMAS).
- The Centre for Academic Information Services has the lawful right to make copies of the Project/Thesis for academic and research purposes only and not for other purposes.
- The Centre for Academic Information Services has the lawful right to digitize the content to be uploaded into Local Content Database.
- The Centre for Academic Information Services has the lawful right to make copies of the Project/Thesis if required for use by other parties for academic purposes or by other Higher Learning Institutes.
- No dispute or any claim shall arise from the student himself / herself neither a third party on this Project/Thesis once it becomes the sole property of UNIMAS.
- This Project/Thesis or any material, data and information related to it shall not be distributed, published or disclosed to any party by the student himself/herself without first obtaining approval from UNIMAS.

Student's signature Felicia
(Date) 30/5/2018

Supervisor's signature: R.
(Date) 30/5/2018

Current Address:
KAMPUNG SUNSURON, PETI SURAT 327, 89657 TAMBUNAN SABAH.

Notes: * If the Project/Thesis is **CONFIDENTIAL** or **RESTRICTED**, please attach together as annexure a letter from the organisation with the date of restriction indicated, and the reasons for the confidentiality and restriction.

[The instrument was prepared by The Centre for Academic Information Services]

Production of Protease from *Aspergillus niger* Under Solid State Fermentation Using Different Agro Waste as Substrate

EDELISA JENNY RADIUS

51650

This project is submitted in partial fulfilment of the requirement of the degree of Bachelor of Science with Honours

(Resource Biotechnology)

Resource Biotechnology (WS47)
Faculty of Resource Science and Technology
University Malaysia Sarawak
2018

Declaration

I hereby declare that this Final Year Project report 2018 entitled **"Production of Protease from *Aspergillus niger* Under Solid State Fermentation Using Different Agro Waste as Substrate"** is based on my original work except for the quotations and citations which have been dully acknowledged. It has not been or concurrently submitted for any other degree at UNIMAS or other institution of higher learning.

Edelisa

Edelisa Jenny Radius

Resource Biotechnology (WS47)

Faculty of Resource Science and Technology

University Malaysia Sarawak (UNIMAS)

Acknowledgements

I would like to express my deepest appreciation towards many people who helped me directly and indirectly to conduct this project. Primarily, I would like to thanks my respected supervisor Miss Rosmawati Saat for her endless support, help, sincere guidance and valuable advices through the completion of this project. I sincerely thank to postgraduate students Ms Shafillah Sulaiman, Mr Nizzal Syafiq Bostaman and Ms Allysya Sylvinessa Alvin for their kindness, help and willing to share their knowledge during the period of my project work.

Special thanks to Prof. Dr Haji Awang Ahmad Sallehin Awang Husaini and Prof. Dr Mohd Hasnain Md Hussain and their postgraduate students Mr Ngieng Ngui Sing, Ms Carolynna Buyau Ak James and Mr Nikson Chong, for permitting and guiding me to get use of the equipment, materials and machine available in Molecular Genetic Lab and Proteomic Lab.

My gratitude also goes to my lab mates Intan Nabihah bt Muhammad Suria Sukma, Shayreen Narisya bt Shaiful Nizam, Nur Fazlin Akmal Bt Muhammad Faizal, Pavithra a/p Madheuan, Shubashini a/p Bakta Vachil and Ravi Kumar Kalaichelvam for their kind cooperation, and to my close friends Stanley Sait ak Agusti, Dymphna Hendra Damin, Rocky Vester ak Richmond, Priscilla Thecla Felix, and Evy Vienney Nelson Donald for their constant moral support and helps in conducting this project.

Last but not least, I dedicated this to both my parents Radius Gandu and Theresa Gadiman and my family for their encouragement, financial support and prayers until this project is completed. Without them, none of this would be possible.

PRODUCTION OF PROTEASE FROM *Aspergillus Niger* UNDER SOLID STATE FERMENTATION USING DIFFERENT AGRO WASTE AS SUBSTRATE

Edelisa Jenny Radius

Resource Biotechnology Programme
Faculty of Science and Technology
Universiti Malaysia Sarawak

ABSTRACT

Proteases are enzymes that catalyse the hydrolysis of protein into polypeptide and oligopeptide to amino acid. These enzymes have various applications in different industries including detergent, leather, and pharmaceutical production. This study focused on the production of protease enzyme from *Aspergillus niger* under solid state fermentation (SSF) using different agricultural wastes including pineapple peel, banana peel and corncob as substrate. This study also investigated the effect of different parameters of solid state fermentation on protease production. Amongst the three different agro waste used as the SSF substrate, pineapple peel recorded the highest protease activity (0.089 U) while the lowest was recorded for banana peel (0.061 U). Therefore, pineapple peel was selected as the substrate of further study on the effect of different SSF parameters. The maximum protease activity obtained from pineapple peel was at 6 days of incubation period, 70 % of initial moisture content and at temperature of 35 °C.

Key words: protease, *Aspergillus niger*, solid state fermentation, agricultural wastes

ABSTRAK

Protease adalah enzim yang memangkinkan hidrolisis protein kepada polipeptida dan oligopeptida kepada asid amino. Enzim-enzim ini mempunyai pelbagai aplikasi dalam industri yang berbeza termasuk pengeluaran detergen, kulit, dan farmaseutikal. Kajian ini memberi tumpuan kepada pengeluaran enzim protease dari Aspergillus niger *melalui fermentasi berkeadaan pepejal dengan menggunakan sisa pertanian yang berbeza termasuk kulit nenas, kulit pisang dan tongkol jagung sebagai substrat. Kajian ini juga telah membuat siasatan mengenai kesan parameter yang berlainan terhadap fermentasi berkeadaan pepejal dalam pengahasilan protease. Di antara ketiga-tiga sisa agro yang digunakan sebagai substrat pada fermentasi berkeadaan pepejal, kulit nenas merekodkan aktiviti protease tertinggi (0.089 U) manakala yang paling rendah dicatatkan untuk kulit pisang (0.061 U). Oleh itu, kulit nenas dipilih sebagai substrat untuk kajian lanjutan terhadap kesan parameter yang berlainan pada aktiviti protease melalui fermentasi berkeadaan pepejal. Aktiviti protease maksimum yang diperoleh daripada kulit nanas adalah pada hari ke-6 tempoh inkubasi, 70 % kandungan kelembapan awal dan pada suhu 35 °C.*

Kata kunci: protease, Aspergillus niger*, fermentasi berkeadaan pepejal, sisa-sisa pertanian*

Table of Contents

List of Tables

List of Figures

List of Abbreviations

A. niger	*Aspergillus niger*
GRAS	Generally Recognized as Save
PDA	Potato Dextrose Agar
rpm	Revolution Per Minute
SmF	Submerged Fermentation
SSF	Solid State Fermentation
TCA	Trichloroacetic Acid
g	Gram
M	Molar
mg/µl	Milligram per microliter
ml	Millilitre
pH	Potential of Hydrogen
US	United State
U/ml	Unit per millilitre
µM	Micro Molar
°C	Degree Celsius
%	Percentage

1.0 INTRODUCTION

Protease refers to the class of enzyme that hydrolyse peptide bond of protein into polypeptide and amino acid (Ahmed *et al.*, 2017). This enzyme represents as one of the largest group of industrial enzymes which have various applications in different industries including detergent, silk, dairy, food and pharmaceutical production (Tunga *et al.*, 2003). Among various sources of protease, microorganisms are commonly utilized due to the low production when using other sources of animals and plants (Haq, 2009). Several types of microorganism such as bacteria, fungi, yeast and Actinomycetes were known to produce protease enzymes (Madan *et al.*, 2002). Amongst these microorganisms, filamentous fungi have been frequently used due to their ability to grow on complex solid substrate and better yield of extracellular enzyme (Nafisa *et al.*, 2017).

Microbial proteases can be secreted in the fermentation medium. Both SmF and SSF techniques can be used to produce microbial enzymes. However, SSF was preferred to be used as it has been reported to have huge potential for the production of enzyme compared to SmF (Ghildyal *et al.*, 1985). Moreover, SSF offer more advantages including utilization of simpler machinery, superior volumetric productivity, cheap and widely available substrate, lower energy requirement and simpler downstream processing compared to SmF (Paranthaman *et al.*, 2009). Ellaiah *et al.* (2002) reported that several species of strain including fungi (*Aspergillus flavus, Aspergillus melleu, Aspergillus niger, Penicillium griseofulvin,*) and bacteria (*Bacillus licheniformis, Bacillus firmus, Bacillus subtilis, Bacillus thuringiensis*) have high ability to produce protease.

It is very important to choose an appropriate substrate for the fermentation process. Different agricultural wastes have been used as substrate for SSF, such as wheat bran, rice bran, maize

bran and sugarcane bagasse (Demain & Solomon, 1996). Agricultural wastes are considered to be the best substrate for SSF process because these substrates act as a carbon source for the induction and biosynthesis of protease (Muthulakshami *et al.*, 2011; Geethanjali & Reshma, 2014).

1.1 Problem statement

Malaysia is one of the countries in the world that produce a large amount of agriculture waste throughout the year. Like most developing countries, Malaysia is facing an increase in the generation of waste and problems associated with waste disposal (Lau, 2004). Approximately, 30, 000 tons of solid wastes are generated daily, which cover 83 % of the country's waste generation including agriculture wastes. According to (Fauziah & Agamuthu, 2009), 95 % of the total wastes are sent to landfills for disposal. This has become a big concern as the waste that being disposed is eliminated through burning which will lead to the environmental pollution. In addition, the industrial activity in our country is elevating rapidly and at the same time the demand of protease enzyme as one of the important industrial enzymes is also increasing. Therefore, the utilization of agro wastes as the substrate for solid state fermentation will reduce the amount of waste being discharge to the environment and at the same time gives a huge contribution to industrial development of enzymes.

1.2 Objective

1. To investigate the effect of different agro waste used as the substrate for the production of protease under solid state fermentation using *A. niger*.
2. To determine the best substrate for protease production by *A. niger*.
3. To identify the optimal SSF condition for protease production by *A. niger*.

2.0 LITERATURE REVIEW

2.1 *Aspergillus niger*

Aspergillus species are saprophytic and are often found in soil and plant decaying material where they secrete digestive enzyme to externally break down organic molecule into more simple nutrients which then can be utilize (Gibbons, 2012). Today these fungi are among the most economically important of the fungal genera (Varga *et al.*, 2008). *Aspergillus* spores are common components of aerosols where they drift on air current due to their small size, normally ranging from 2 to 3 μ (Latge, 1999). They disperse themselves on short and long distance depending on their environmental condition. When the spores contact with solid or liquid surfaces, they are deposited and will germinate if the conditions of moisture are right (Kanaani *et al.*, 2008). The genus *Aspergillus* is also a group of filamentous fungi which today consists of more than 250 species (Varga *et al.*, 2008).Though many microbes can grow on solid substrate, only filamentous fungi have the ability to grow to a significant extent without the presence of free water and can even penetrate within the solid substrate (Kumar, 2002).

Aspergillus species secrete and produce various types of enzymes to degrade organic matter under natural environment (Gibbons *et al.*, 2012). They have the ability to secrete enzyme and acid into the surrounding environment, breaking down polymer molecules into simpler one that are then absorbed back into the fungal cell. Just like animal, fungi are heterotrophic. They gain access to nutrients by mechanical forces whereby fungal hyphal tips grow into and through their food substrate. Leong *et al.* (2006) stated that the optimum growth temperature for *A. niger* is between 35-37 °C. According to Baker *et al.* (2008), the genera *Aspergillus* sp. is highly potential for production of enzymes that could be used to convert plant biomass into fuels and other industrially useful products. Nowadays, many enzymes are produced by

Aspergillus are used in the food and brewing industries, the processing of animal feed and the paper and pulping industry (Asai *et al.*, 2005). Punt *et al.* (2012) stated that among the type of *Aspergillus* used in biotechnology industry, *Aspergillus niger* are the most frequently used in the production of heterologous protein in very high yield. **Table 1** represent list of some common enzymes found from different species of microorganisms.

Table 1: List of some common enzymes found from different species of *Aspergillus* sp

Source	Enzyme	Microorganism	References
Fungal	Amylase	*Aspergillus oryzae*	Akhter *et al.* (2011)
	Glucosidases	*Aspergillus flavus*	Khan *et al.* (2007)
	Proteases	*Aspergillus niger*	Sabir (2007)
	Pectinases	*Aspergillus niger*	
	Catalase	*Aspergillus niger*	

2.2 Protease

Proteolytic enzymes, also called proteases are multipurpose enzymes that catalyse the hydrolysis of proteins into polypeptides and oligopeptides to amino acids. These enzymes are one of the essential groups of industrial enzymes accounting almost 60 % of the global enzyme market due to their various applications in different industries such as detergents, leather, and pharmaceutical production (Abraham *et al.*, 2014). Protease also plays an important role in many industrial processes including animal feed, cheese and food processing (Kumar *et al.*, 2002). Kirk *et al.* (2002) described those proteases enzymes are highly required in baking, brewing and production of oriental foods such as miso, soy sauce, meat tenderization and cheese manufacture.

The source of protease consists of plant, animal tissue and microorganism. Amongst these sources, microorganisms which have broad biochemical diversity and susceptible to genetic manipulation are rapidly used to produce protease enzyme compared to the other two sources that only produce low amount of protease (de Castro *et al.*, 2015). Microorganisms that are commonly used to produce protease enzyme include bacteria, fungi, Actinomycetes, and yeast (Madan *et al.*, 2002).

Schuster *et al.* (2002) reported that *Aspergillus niger* are very useful in protease production, as they are considered "Generally Recognized as Save (GRAS)" by the US Food and Drug Administration. Both SmF and SSF techniques could be used to produce microbial protease. SSF is the most effective technique to produce protease because the solid substrate will act like a natural habitat for the fungi and improve their growth (de Castro *et al.*, 2015). Moreover, the use of SSF technique is very simple, not expensive, produce greater yield and concentration of extracellular enzymes, and the source of the solid substrate can be obtain easily from any suitable agricultural wastes (Chutmanop *et al.*, 2008).

2.3 Solid State Fermentation (SSF)

According to Mitchell *et al.* (2006), solid state fermentation involves the growth of microorganism on moist solid particle, in situation where the spaces between the particles contain a minimum of visible water and continuous gas phase. Even when there is a present of thin films of water at the particle surfaces, the inter-particle water phase is discontinuous and mostly the spaces are filled by the gas phase (Mitchell *et al.*, 2006). Majority of the water in the system is absorbed within the moist solid particle **(Figure 1)**.

Michael *et al.* (2001) described that raw materials used in solid state fermentation usually received pre-treatment to facilitate the fungal attack including grinding or milling to increase the surface area, soaking to soften, and steaming to kill and eliminate resistance associated with living seeds. Hence, the volume of solid substrate used in SSF must be small, otherwise there would be a problem distributing oxygen throughout the fermentation and in dissipating the carbon dioxide and heat generated by fungal metabolism. Fungal extracellular enzyme that involved in SSF break down polymers and further microbial processing yields nutritious and palatable products such as miso, soy sauce and tempeh (Michael *et al.*, 2001). SSF have many merits compare to SmF. The solid substrates are widely available and cheap, sterilization is not usually needed and the energy requirements are low. Moreover SSF only use simpler machinery and downstream processes (Paranthaman *et al.*, 2009).

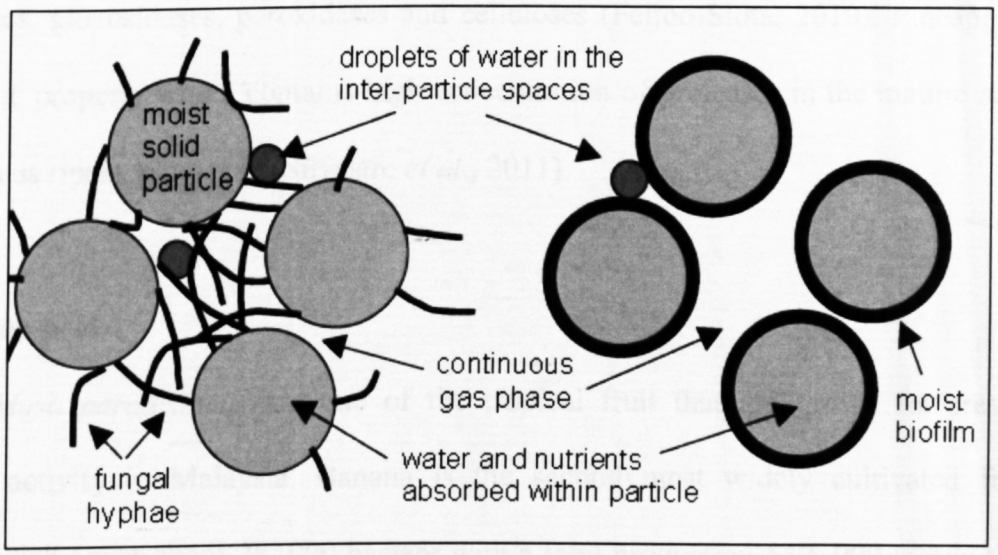

Figure 1: The arrangement of moist solid particles and the continuous gas phase in SSF system involving a filamentous fungus (left hand side) and a unicellular organism (right-hand side) (Mitchell, 2006)

2.4 Agro wastes

2.4.1 Pineapple peel

Pineapple (*Ananas comosus*) is one of the popular tropical fruit crops among the fruits crop produced and cultivated globally (Singh *et al.*, 2003). These fruits are commonly grown and yield the best in the areas with warm and relatively uniform climate year around (Singh *et al.*, 2008). In Malaysia, pineapple plant is widely cultivated for its fresh consumption, canning and juicing. Increasing of pineapple plantation also raise the pineapple waste proportionally, in a massive amount (Yusri *et al.*, 2016). Pineapple peels are one of the parts of this fruit that represent as a huge amount of waste product from the pineapple industry (Cesar, 2005). Hebbar *et al.* (2010) stated that pineapple is the best known source of proteolytic enzyme bromelain compared to the other plant family *Bromeliaceae.* Pineapple peel contains bromelain that constitute of a mixture of various proteases as well as carbohydrates, phosphatases, glucosidases, peroxidases and celluloses (Feijoo-Siota, 2010). Pineapple also has a unique property which contains high concentration of proteases in the mature stage or also known as ripens pineapple (Silvestre *et al.*, 2011).

2.4.2 Banana peel

Bananas (*Musa paradisiaca*) are one of the tropical fruit that are grown for fresh and processing activity in Malaysia. Banana is the second most widely cultivated fruit in Malaysia which cover about 26, 000 hectare with a total production 530, 000 metric tonnes. The growing system of banana in Malaysia are as Monocrop and Mixed Planting System and known as one of the largest fruit tree grown after durian, pineapple, rambutan and papaya. This fruit contain various nutrients such as fibre, Vitamin B and C. Usually people only consumed the fleshy part of banana especially when it is ripen. The processing of banana into products mostly bakery foods such as biscuits, breads, cakes, puree, chips, jam, juice, ice

Table 2: Chemical composition of different agro waste

Agricultural wastes	Chemical composition	References
Pineapple peel	37.49 % Carbohydrates 14.80 % Crude fibres 5.31 % Lipids 5.11 % Crude protein 4.39 % Ash	Feumba *et al.* (2016)
Banana peel	32.39 % Carbohydrate 5.53 % Protein 0.884 % Nitrogen 13.49 % Moisture 23.93 % Lipid 14.83 % Fibre 9.83 % Ash	Abubakar *et al.* (2016)
Corn cob	48.56 % Carbohydrate 4.19 % Protein 0.78 % Nitrogen 6.00 % Moisture 4.72 % Lipid 33.33 % Fibre 2.49 % Ash	Abubakar *et al.* (2016)

3.0 MATERIALS AND METHOD

3.1 Preparation of *Aspergillus niger*

Aspergillus niger was obtained from Molecular Biology Lab fungal collection, FRST, UNIMAS. The fungus was sub-cultured and maintained on Potato Dextrose Agar (PDA) with ampicillin (50 mg/µl) and incubated for 4-6 days at room temperature.

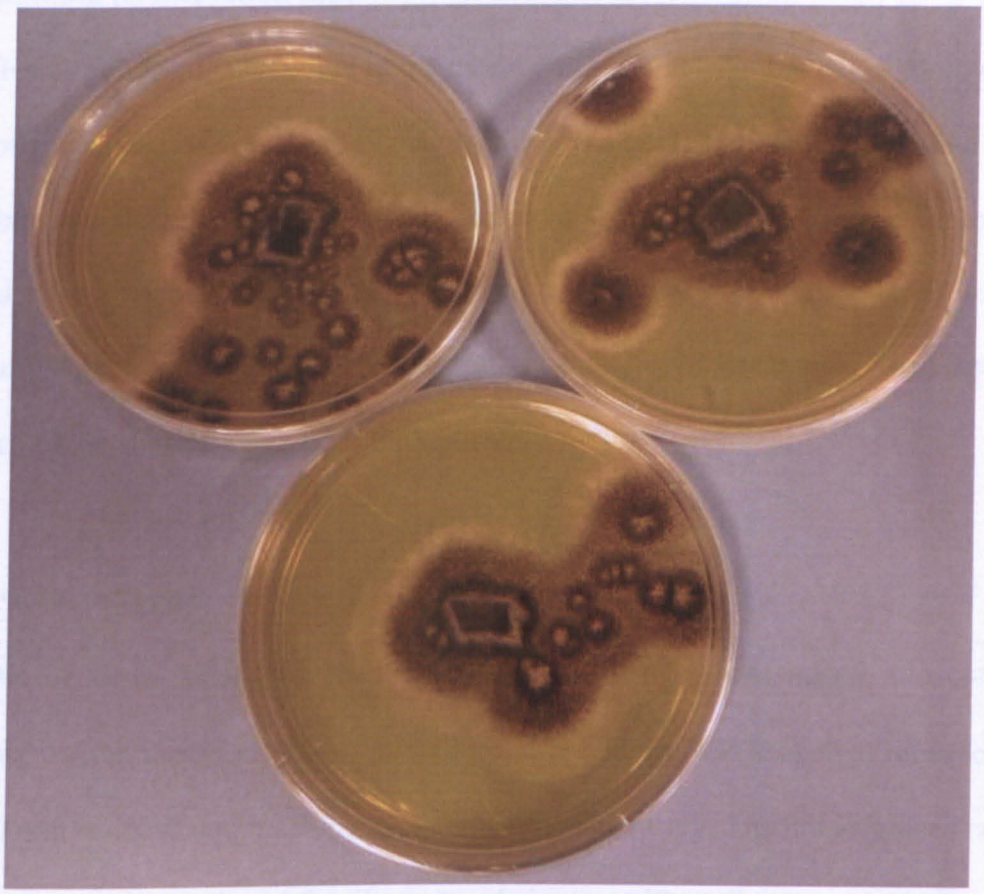

Figure 2: Formation of black spores and white spores on the *A. niger* strain during day 3

3.2 Preparation of substrates

Pineapple peel, banana peel and corncob were use as substrates for the solid state fermentation. All these three substrates were obtained from Bandar Riyal market and Farley Supermarket Kota Samarahan. The pineapple peel was washed under running tap water to

remove any debris, chop into small pieces, dried up in an oven at 70 °C for 2 days, and then the dry biomass was ground (Olaniyi *et al.*, 2013). This method was repeated by using banana peel and corncob. All of the three substrates which have been pre-treated mechanically were dried up in an oven with 60 °C until constant weight is achieved and the existing moisture content is determined.

3.3 Solid State fermentation (SSF)

The SSF was conducted according to the modified (Muthulakshmi *et al.*, 2011) method. Five grams of pineapple peel was added into a 250 ml conical flask. Sterile distilled water was added into the flask to obtain 70 % of initial moisture content. Three plugs of *A. niger* obtained from previous culture was inoculated on the substrate and the flask was covered with aluminium foil. The process was repeated using 5 g of banana peel and 2.5 g of corn cob. Each of the substrate was done in duplicate.

3.4 Extraction of crude enzyme

A volume of 20 ml of 0.1 M sodium acetate with pH 5.8 was added into conical flask containing the fermented substrate and culture. The mixture was shaken at room temperature with 120 rpm using shaker machine available in the laboratory. The homogenised culture was filtered using muslin cloth and the filtrate was being centrifuge directly at 6,000 rpm, 4 °C for 30 minutes. The supernatant result from the centrifugation was being filtered through filter paper twice. The clear supernatant was subjected to enzyme assay.

3.5 Protease enzyme assay

Protease activity was determined according to Ahmed *et al.* (2017) method. This was accomplished by adding each enzyme extract (1 ml each) and 2.0 ml of 0.5 % casein solution into a test tube and the tubes was incubated at 35 °C for 10 minutes in a water bath. The residual protein was precipitated by adding 3.0 ml of 10 % ice trichloroacetic acid (TCA). The precipitates was allowed to settle for one hour and then centrifuged at 5,000 rpm for 5 min. One millilitre of each supernatant was mixed with 5 ml of 1 M sodium carbonate. After 20 min, 0.5 ml of Folin and Ciocalteu's phenol reagent was added. The concentration of liberated tyrosine in filtrate was measured at 660 nm against a reagent blank using tyrosine standard (Lowry *et al.*, 1951). Standard curve was prepared using following concentration range of tyrosine: 0.055, 0.111, 0.221, 0.442 and 0.553 µM **(Appendix C)**. One protease unit (U) is defined as the amount of enzyme required to releases 1 µM of tyrosine per minute per ml (Mohapatra *et al.*, 2003). All the experiment was done in duplicate and mean values was presented. Protease activity was determined using the following formula:

$$= \frac{\mu\text{mole tyrosine equivalent releases} \times \text{total volume of assay (ml)}}{\text{Volume of enzyme taken (ml)} \times \text{incubation (min)} \times \text{measured sample volume (ml)}}$$

3.6 Optimization of SSF parameters

The effects of several parameters constitute initial moisture content, temperature and incubation time on SSF were optimised to obtain optimum protease activity. Amongst the three different types of agro wastes, pineapple peel which has recorded the highest protease activity was selected and used as substrate to study the optimization parameters.

3.6.1 Effect of incubation period

Effect of incubation time on protease production was studied by looking at different SSF incubation period; 48 hours (2 days), 96 hours (4 days), 114 hours (6 days), and 192 hours (8 days). The temperature was adjusted to 30 °C and 70 % of moisture content.

3.6.2 Effect of initial moisture content on SSF

The initial moisture content of the substrate was adjusted to 60 %, 70 %, and 80 % with sterile distilled water. The cultivation parameters were 6 days of incubation period and temperature set at 30 °C.

3.6.3 Effect of incubation temperature on SSF

The effect of incubation temperature was determined by performing the SSF process at different temperatures. In this study the range of 25 °C, 30 °C, 35 °C, and 40 °C incubation temperature was studied. The SSF was performed with 70 % of moisture content and 6 days of incubation period.